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Immune memory responses to HBV vaccine 13-18 years after primary vaccination

L. Hou¹, W. Li¹, X. Wei², Y. Zhou², Y. Zhuo², H. Wu¹ and B. Shen¹

¹Department of Infectious Diseases I,
The First Affiliated Hospital of Xinxiang Medical University,
Weihui, Henan Province, China

²Department of Infectious Diseases II,
The First Affiliated Hospital of Xinxiang Medical University,
Weihui, Henan Province, China

Corresponding author: B. Shen
E-mail: baoshengshen@163.com

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ABSTRACT. The aim of this study was to evaluate the immune memory response 13-18 years after an hepatitis B virus (HBV) vaccine by performing a specific *in vitro* stimulation experiment. Thirty healthy volunteers who had been inoculated 13-18 years ago with the HBV vaccine were collected from the physical examination center. Peripheral blood mononuclear cells were stimulated with 50 ng/mL recombinant HBsAg. An ELISA kit was used for the detection of antibodies that were produced by these cells *in vitro*. It was found that even 13-18 years after inoculation with the HBV vaccine, an anamnestic antibody response still existed, and was not correlated with the serum antibody levels ($r = -0.177$, $P = 0.377$). In conclusion, our data showed that the individuals after inoculation, including those with anti-HBs <10 IU/L as well as those individuals in whom the antibody was not detected, retained immune memory, which may be the main role of memory B cells.

Key words: Hepatitis B virus (HBV); Vaccine; Immune memory

INTRODUCTION

A vaccine can be the most economical and effective means of preventing the spread of infectious diseases. The mechanism of the action of a vaccine is the induction of a protective immune response. The characteristics of immune memory are having the selective and rarity. A secondary immune response to the same antigen can induce the production of stronger antibodies as compared to the primary vaccination, and this is called immune memory. The phenomenon of immune memory can occur regardless of whether the humoral or cellular immune response is activated. In humoral immunity, the secondary immune response to TD antigen shows significantly increased antibody titer. Moreover, during this response, the immunoglobulin can be converted from IgM to IgG, and with this change, the avidity of the antibody is enhanced. This suggests that the secondary immune response not only involves a quantitative change in the amount of antibody produced, but also a change in the quality of the antibodies produced.

Experiments proved that the basis of immune memory was the generation of immune memory cells. The humoral immune response is typically associated with specific pathogens, such as hepatitis B virus (HBV) and hepatitis A virus (HAV), which can be neutralized by existing antibodies. These antibodies are generated by the plasma cells, which then secrete the antibody (Lanzavecchia et al., 2006). The half-life of an antibody *in vivo* is only a few days, and thus, to maintain a specific antibody titer the plasma cells must continuously secrete antibodies (Manz et al., 1998). The activation of an antigen can induce B cells to differentiate into plasma cells. The process needed interaction with helper CD4⁺ T cells. In order to provide the expression and signal transduction of cytokines, CD4⁺ T cells can recognize the peptide antigen expressed by antigen presenting cells (APCs). In the immune response, follicular dendritic cells (FDCs) were the strongest APCs. Research has shown that long-lived plasma cells can allow the immune system to maintain a stable immunological memory (Manz et al., 2005). The specific antibody titer against a particular antigen declines after several years; however, FDCs release more antigens to stimulate memory B lymphocytes to compensate for the decreased number of plasma cells (Slifka and Ahmed, 1998).

In this study, we aimed to assess the HBV humoral memory response of inoculated individuals by an *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) with recombinant hepatitis B surface antigen.

MATERIAL AND METHODS

Subjects

Thirty healthy volunteers (24 female and 6 male, aged 13-60 years) were included in this study. All the volunteers had obtained the standard three doses of the HBV vaccine in the past 13-18 years according to their inoculation card. They were divided into three groups according to the concentration of HBV antibodies (anti-HBs) in their serum: group I: serum anti-HBs were not detected (N = 10); group II: serum anti-HBs <10 IU/L (N = 15); group III: serum anti-HBs >10 IU/L (N = 5). The recombinant hepatitis B vaccine rHBV-IB (Kangtai Biotechnology Co. Ltd., Shenzhen, China) was used for lymphocyte stimulation *in vitro*. This monovalent vaccine was produced by 20 µg purified recombinant yeast with aluminum hydroxide as an adjuvant. This study was conducted in accordance with the Declaration of Helsinki. This study was also conducted with approval from the Ethics Committee of the First

Affiliated Hospital of Xinxiang Medical University. Written informed consent was obtained from all participants.

Detection of serum anti-HBs

Blood samples of all participants were collected for the detection of HBsAg. An ELISA kit (Sigma-Aldrich, St. Louis, MO, USA) was used for detecting serum anti-HBs. All experimental procedures were performed according to the manufacturer instructions.

Lymphocyte stimulation test

The optimal conditions for lymphocyte stimulation are presented in Livramento et al. (2013). The PBMCs (Sigma-Aldrich) were isolated and purified using lymphocyte separation medium and heparinized venous blood. PBMCs were resuspended with DMEM medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco), 200 mM L-glutamine, 10 mM HEPES, 110 mg/mL sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin. PBMCs were then distributed into the wells of a 96-well cell culture plate at 4×10^6 cells/mL. The sample was stimulated with 50 ng/mL HBsAg under culture conditions at 37°C and 5% CO₂ according to a previously described method (Wang et al., 2004). Cells were stimulated for 3 days and anti-HBs were detected by an ELISA quantitative detection kit (Sigma-Aldrich) in the media of the culture solution.

Avidity detection of anti-HBs by induction *in vitro*

After the cells were stimulated for 3 days, we assessed the avidity of anti-HBs secreted by the cells using a modified anti-HBs ELISA kit (Biokit, Barcelona, Spain), in which urea incubation steps were introduced. The culture plate was incubated with 100 µL cell culture media at 37°C for 1 h and then washed with the lotion three times. Dissociation buffer was then added (100 µL) to each well in row A (8 M urea and PBS containing 0.05% Tween-20, pH 7.2) for incubation. PBS not containing urea was added (100 µL) to row B, and 30 min later, the sample was washed with lotion. Lastly, 100 µL anti-human secondary antibodies that were linked to alkaline phosphatase was added to each of the aforementioned wells and washed with lotion for 30 min at 37°C. The TMB substrate containing hydrogen peroxide was then added to the above solutions for 30 min. Following the incubation, 50 µL 1 M HCl was added to terminate the reaction. OD was measured at 450/630 nm with a microplate detector, and purified anti-HBs were taken as the positive control.

Calculation of affinitive index

The avidity index is expressed as a percentage, and is the OD ratio of signal with urea and without urea, which is then multiplied by 100. AI < 30% was considered to indicate an antibody with low avidity (Namujju et al., 2011; Yamada et al., 2011).

Statistical analysis

All data were analyzed using the SPSS17.0 statistical software (SPSS Inc, Chicago,

IL, USA). The data of two or more groups were compared with the Student *t*-test or ANOVA analysis. The correlation between parameters was analyzed by Pearson's linear regression, $P < 0.05$ was considered to be statistically significant.

RESULTS

Detection of HB-specific antibodies in cell culture medium

A Sandwich ELISA was used to evaluate the PBMC-derived secretion of HB-specific antibodies in cell culture medium following stimulation of lymphocytes with 50 ng/mL HBsAg. Of the thirty cell culture media tested, 27 contained detectable levels of anti-HBs (Figure 1A). The antibody group, namely group I, was not detected in the serum. The amount of anti-HBs produced by vaccine stimulation *in vitro* was significantly lower than that of serum anti-HBs inoculator (<10 IU/L) (Figure 1C). On the other hand, the linear regression analysis showed that the level of anti-HBs secreted within the cell culture media was not positively correlated with the level of anti-HBs in the serum ($r = -0.041$, $P = 0.829$).

Figure 1. Antibody responses *in vitro* of vaccinated individuals on recombinant HBsAg stimulation. The supernatant was cultured by ELISA. The amount of anti-HBs (A) and experiment results of affinity of the antibody secreted *in vitro*. B. Slope on serum antibody; C. three groups of vaccinated individuals, antibody in the supernatant secreted by HBsAg induced PBMCs *in vitro*; D. affinitive indexes of anti-HBs secreted in three groups of vaccinated individuals *in vitro*.

Avidity of antibodies to HBsAg

The avidity of antibodies to HBsAg was variable (Figure 1B). However, variation

analysis showed that there was no significant difference in the avidity indexes between the three groups ($p > 0.05$) (Figure 1D). Indeed, when group II and group III were compared, the results indicated that the antibodies had similar affinitive maturity (respective values: 33.2 ± 22.7 , $33.7 \pm 18.9\%$; $P = 0.964$) (Table 1). The avidity index of anti-HBs in cell culture media *in vitro* had no significant correlation with anti-HBs titer in serum ($r = -0.177$; $P = 0.377$).

Table 1. Humoral immune response in the three groups of vaccinated individuals *in vitro* on HBsAg stimulation.

Serum anti-HBs (group)	Time from vaccination to test (years)		<i>In vitro</i> anti-HBs secretion	Amount of anti-HBs secreted <i>in vitro</i> (IU/L)		Avidity index of anti-HBs secreted <i>in vitro</i> (%)		High-avidity result ($\geq 30\%$)
	Mean	95%CI		Mean	95%CI	Mean	95%CI	
Undetectable	15.7	14.4-17.0	8/10 (80.0)	10.6	5.8-15.4	55.0	36.3-73.7	6/10 (60.0)
<10 IU/L	15.9	14.7-17.0	14/15 (93.3)	23.8	15.9-31.8	33.2	20.1-46.3	6/15 (40.0)
≥ 10 IU/L	16.8	14.6-19.0	5/5 (100)	17.6	10.8-24.5	33.7	10.2-57.2	3/5 (66.7)

CI = confidence interval.

Avidity index of HBsAg antibody

According to the avidity index of the HBsAg antibody, the critical value of affinitive ratio was 30%, and can be used to distinguish samples (Table 1). The specific immune responses induced by HBsAg memory cells indicated that the antibodies possessed a high avidity for HBV *in vitro* (more than 30%).

DISCUSSION

After antigen stimulation, serum antibody levels reached their peak. The antibody titers then decreased but then stabilized at some level that was maintained for several years. Indeed, individuals inoculated with the HBV vaccine showed a decrease in the level of antibodies produced over time. Generally, 1 month after the HBV vaccination (involving the injection of 3 needles), the surface antibody could be detected in 97% of the people, and this level remained the same in the second year. In the third year, detection dropped to 74% of people and the antibody titers also decreased. The long-term maintenance of immune memory is highly advantageous as it can provide an immediate response to pathogens to which the host has been previously exposed. Despite the level of anti-HBs after inoculation with three doses of the HBV vaccine (<10 IU/L; Livramento et al., 2011; Scaraveli et al., 2011; Tonial et al., 2011), the implementation of the universal HBV vaccination program significantly reduced the incidence of HBsAg carriers and HBV-related diseases (Chongsrisawat et al., 2006; Tosun et al., 2011). The survival of a memory cell has the potential to continuously stimulate the antigen. However, it remains unclear as to whether it is necessary to improve the antigen dose during the initial HBV vaccination, or whether a second inoculation is necessary for those whose serum levels of the antibody are undetectable.

In this study, the recombinant HBsAg was used to stimulate PBMCs (from previously inoculated subjects) to produce antibodies *in vitro*. The subjects involved in this study included people who possessed low-level serum anti-HBs (<10 IU/L) or whose anti-HBs were not detected. Moreover, utilizing the culture media from PBMCs that had been stimulated by antigen activation is a viable way to detect anti-HBs with high avidity, which proves the

existence of a memory response. The existence of antibodies that have a high avidity for their antigen proved that recombinant HBsAg can induce the humoral memory response (Joseph et al., 2001; Alam et al., 2013). However, the memory response to HBsAg was more difficult to evaluate. The reasons for this difficulty were the different experimental methods, different manufacturers of vaccines, and exposure to different concentrations of HBV during the study period (Poovorawan et al., 2010). The results of this study showed that 40% of the patients in whom the antibody level in the serum was lower than 10 IU/L and 60% of those who lack serum antibodies *in vivo* respectively showed immune memory response. The present result also proved that the humoral immune response of inoculated individuals, as determined by the antigen stimulation of PBMCs, was not related with antibody titer in the serum, which was consistent with previous observations (Leyendeckers et al., 1999; Rosado et al., 2011). On the other hand, assessment of the immune response to HBsAg *in vivo* showed a positive correlation between previous high antibody titer and memorial anti-HBs response (Su et al., 2007; Eldesoky et al., 2009). The vaccinated individual with a low serum titer of anti-HBs cannot be shown to produce a memory response to a high dose of antigen. Whether these individuals are susceptible to HBV is still unclear, however a deeper epidemiological investigation may shed light on this issue.

In summary, our results indicated that the PBMCs of previously inoculated individuals could still produce antibodies after antigenic stimulation *in vitro*. The memorial antibody response was observed in all vaccinated groups, including those where no or low concentrations of antibody were detected in serum. However, in order to determine the reaction in the absence of memory response, the susceptibility to HBV for the inoculators needs a follow-up study to decide whether to increase the antigen dose after the primary vaccination. This may provide protection to those inoculated individuals that possess anti-HBs below 10 IU/L. In our country, it is generally believed that those titers of hepatitis B surface antibody less than or equal to 10 IU/mL should be vaccinated within 6 months. Antibody titers greater than 10 IU/mL can be re-inoculated within 6 years.

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RETRACTION